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DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in the
Faculty of Pure Science of
Columbia University.

BY

JANE ELIZABETH DALE

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THE INFLUENCE OF SALTS UPON THE HYDROLYSIS OF STARCH BY PANCREATIC AMYLASE

Neutral salts have long been credited with an activating effect upon amylases. Nasse[1] in 1875 found that the chlorides, nitrates and sulphates of sodium, potassium and ammonium increased the saccharogenic power of amylases and he formed the hypothesis that an important relation exists between enzyme activity and the presence of salts.

Vernon[2] found that the addition of small amounts of salt increased the activity of pancreatic extracts and also diminished the reduction of activity occassioned by dilution.

Preti[3], working with urine, blood serum and pancreas preparations, rendered them ineffective as amylases by dialysis and restored their power by adding sodium chloride. Potassium, magnesium and calcium chlorides, and sodium and potassium bromides, iodides, fluorides, sulphates and nitrates were also effective in reactivating dialyzed pancreatin or malt.

Kendall and Sherman[4] concluded, from experiments in which pancreatin acted upon either dialyzed or undialyzed starch solutions in the absence of added salts or in the presence of sodium, potassium or ammonium chlorides, that salt not only helps the diastatic action but is essential to it. The salts accidentally present in commercial pancreatin and in very carefully prepared soluble starch are sufficient to enable the amylase to act to some extent, but any results so obtained are abnormal and entirely misleading as indications of real diastatic power, since pancreatic amylase acts in nature in a medium containing salt and must have an appreciable amount of some salt in order to function normally.

Bierry[5] found that the alkaline and alkaline-earth chlorides and the alkaline bromides restored the activity of dialyzed pancreatic extract in part at least. He believed that only a portion of the activity was regained because part of the amylase dia-

lyzed away and part was held by the collodion membrane. Similar results were obtained for amylases from saliva and intestine, from which Bierry concluded that halogens are necessary for the activity of animal amylases.

Michaelis and Pechstein[6], Groll[7], and Biedermann and Rueha[8] confirmed the findings of Bierry for salivary amylase and halides. The former investigators believed that sodium sulphate was without effect upon the salivary hydrolysis and that sodium nitrate inhibited the action; their conclusion was based upon comparison of hydrolyses in the presence of varying amounts of sulphate and nitrate with hydrolyses in the presence of N 0.02 sodium acetate as practically salt-free. They found that the inhibiting action of sodium nitrate was noticeable down to a concentration of N 0.001; below that point the activity was the same as for the "salt-free" control. Groll[7] and Biedermann[9] both reported that nitrates and sulphates inhibited the action of the amylase.

Crude malt extract apparently does not require additional electrolyte for activity. Bierry[5] found this true for dialyzed malt extract as well. Osborne[10] however found that his highly purified malt amylase did not exert its true diastatic power unless sodium chloride was added. Similarly Sherman and Thomas[11] increased the activity of purified malt amylase by adding chloride, sulphate or nitrate of sodium or potassium to their starch preparations.

In experiments such as those outlined above a neutral salt may function simply as an electrolyte increasing the total ionic concentration of the starch-enzyme solutions. Various theories have been advanced as to the effect of this increase. Arrhenius[12] in 1889 assumed the existence in solution of equilibrium between active and inactive forms of substrate, and suggested that increase in ionic concentration displaced the equilibrium in the direction of an increase of the active modification, thereby resulting in increase of reaction velocity.

Le Blanc[13] in 1891 came to the conclusion that the dissociation of water is increased by the addition of salts. Euler[14] in 1900 advanced the hypothesis that in hydrolyses water acted by means of its ions and that salts cause the well-known salt

catalysis because they effect a corresponding change in the dissociation constant of the water.

Arrhenius[15] found an increase in the rate of inversion of sucrose by weak acids upon the addition of neutral salts, and concluded that the dissociation constants of weak acids and of weak substances in general are greatly enhanced by the mere presence of strong electrolytes.

Acree[16] made a detailed study of the effect of salts and concluded that "salt catalysis is a very complex phenomenon caused by a combination of effects, a minor one of which may be that assumed by Euler. Catalyzing salts may combine with the reacting salts or substances and form still more (or less) reactive double salts or double compounds." They may cause changes in solvation, viscosity, heat of dilution, vapor tension, and osmotic pressure. They may have a "concentrating effect due to the union of the solute with the solvent. Finally . . . the thermodynamic potential or 'activity' or driving force behind each reaction depends not only upon the substances themselves but upon the surrounding field. When this is changed by the addition of electrolytes or non-electrolytes, there will be a change in the corresponding thermodynamic potential and therefore in the reaction velocity."

McBain and Coleman[17] criticized the hypothesis that neutral salts increase the dissociation of weak acids and bases. Their criticism was based on the work of Bredig, Acree, Snethlage and Taylor which indicates catalysis by undissociated molecules of electrolytes as well as by ions. In 1919 McBain and Karn[18] attributed the effect of salts to increase in chemical potential of undissociated molecules and of ions. This had been the view taken by Harned [19] in 1915 in regard to hydrogen and hydroxyl ion activities of solutions of hydrochloric acid and sodium and potassium hydroxides in the presence of neutral salts. Brönsted [20, 21] has made a detailed study of the effect of salts on activity coefficients.

A radically different effect of salts has been suggested by Hahn and Michalik [22], who believe that neutral salts affect the colloidal character of enzymes.

Starkenstein[23] favored the view of compound formation between the activating salt and the enzyme and attempted to show a definite relation between their concentrations. Michaelis[24] assumed a combination of enzyme with substrate and later[25] stated that this enzyme-substrate compound acted as a weak base or acid and formed ionizable salts with ions present in the solution. Ambard[26] believed that enzyme action is preceded by the formation of a complex between enzyme and substrate which takes place in the presence of electrolytes only.

It will be seen then that a neutral salt may function in the enzymic hydrolysis of starch either by some specific effect of one or both of its characteristic ions on starch or enzyme, or starch-enzyme complex, or by secondary effects due to its ionic concentration. Not the least of these is the possible increase of concentration or activity of the other ions in the experimental solutions. The alkaline salts of weak acids may function in these ways and may, in addition, render the reaction of the solutions more alkaline or, in the case of solutions which have been brought to a definite hydrogen-ion concentration, protect by their buffer action against changes in hydrogen-ion concentration during the course of experiments.

An attempt has been made in this investigation to throw further light upon the rôle of salts in the hydrolysis of starch by enzymes. Four typical alkaline salts of weak acids, sodium acetate, sodium borate, sodium citrate and sodium phosphate, have been chosen for study. Carefully controlled, parallel hydrolyses of starch dispersions containing the four salts were planned in the belief that they would proceed to different degrees of sugar formation if the characteristic anions exerted any specific activating or inhibiting effect upon the activity of the amylase.

Many references to the anions of all these salts except the borate are found in the literature. Unfortunately early work did not take into account the hydrogen-ion concentration of experimental preparations. For instance, Detmers[27] reported a slight increase in the amyloclastic action of malt extract upon addition of citric or phosphoric acids. Neilson and Terry[28] activated liver diastase with sodium citrate in determining the

rate of transformation of glycogen to dextrose. Schneidewind, Meyer and Müntner[29] found acetic and citric acids favorable to diastatic activity at a concentration of 0.001% but injurious at 0.01%; very small additions of acid phosphate had a favorable effect.

In 1909 Sörensen[30] published the results of an extended study of the determination of hydrogen-ion concentration and of its effect upon enzymic hydrolysis. He concluded that the activity of an enzyme is a function of the hydrogen-ion concentration of the medium in which it acts and that it is independent of the anion of the acid used. His own work was done with catalase, pepsin and invertin tested with hydrochloric, sulphuric, phosphoric and citric acids. Furthermore he found that a definite relation exists between three factors, the temperature and the time of the hydrolysis and the hydrogen-ion concentration of the medium. Within fairly narrow limits the optimum hydrogen-ion concentration varies with the temperature and the time; similarly the optimum temperature will vary with the time and the hydrogen-ion concentration. Therefore it is absolutely necessary that investigators reporting optima should state all experimental conditions. This has not always been done and therefore the results of studies of salt effect are not easily interpreted.

Sherman and Thomas[11] extended Sörensen's findings in work with malt amylase. They found that acetic, propionic, phosphoric, hydrochloric, nitric and sulphuric acids, and the primary phosphates of sodium and potassium increased the activity of purified malt amylase to the same extent, and that the optimum concentration with each electrolyte was that which gave a pH value of 4.2 to 4.6 by electrometric determination.

A few investigations which have been conducted with various amylases acting in media of definite hydrogen-ion concentration fail to show consistent results with acetates, citrates and phosphates. Michaelis and Pechstein[6] used acetate and phosphate mixtures primarily for maintenance of hydrogen-ion concentration and found that at the same concentration (optimum pH 6.1—6.2) the activity of ptyalin in the presence

of each "buffer" was quantitatively the same. No appreciable difference was found when the phosphate concentration varied between M/200, M/100, M/50, M/25 and M/5. Later experiments with sodium acetate showed a slight depression of activity as soon as the concentration was increased beyond N/10. The optimum was determined for amyloclastic activity by a method essentially that of Wohlgemuth; pH was determined electrometrically.

Ringer and van Trigt [31] confirmed the finding of the same activity for ptyalin at the same optimum pH in the presence of phosphate and acetate. Their optimum for saccharogenic activity, pH 6.0, was determined electrometrically at 18°. The optimum was independent of the phosphate concentration. In contrast they found that with citrate the optimum pH was higher and increased with increasing concentration of the "buffer." Less sugar was formed absolutely and also in relation to the acidity of the medium since the optimum of activity was shifted toward the alkaline side.

Hahn and Michalik [22] have found more recently that the optimum pH for amyloclastic activity of both salivary and pancreatic amylases was decreased when acetate mixture was substituted for phosphate mixture in the starch-enzyme preparation.

In the case of urease Van Slyke and Zacharias[32] reported a specific "salt effect" of the phosphates. They found that, while neutral phosphate mixtures can maintain the enzyme action near its maximum rate by preventing the development of alkalinity, the phosphates also exert a retarding effect on the action . This retardation increased with increasing phosphate concentration and was independent of hydrogen-ion effect. As the lowest concentration of phosphate used in their experiments was M/21 it is quite possible that their "salt effect" was the more general tendency of electrolytes to "salt out" colloids which has since been recognized.

Because of these conflicting reports it was decided that a study of the influence of the four alkaline salts above mentioned must include a determination of the optimum pH for sugar formation in starch dispersions which contained each of the

salts at the same comparatively low molar concentration. Determination of the effect of variation in salt concentration upon the enzymic activity and upon the optimum pH was also necessary. After this preliminary study it would be possible to compare the effects of the salts at the same concentrations and the same pH and also at those concentrations and pH values which were found to be best for each salt. It was expected that such a comparison would show definitely whether or not the characteristic anions of the salts exert any specific influence, either activating or inhibiting, upon the starch hydrolysis.

EXPERIMENTAL

Materials. Pancreatic amylase was chosen as the enzyme for study since the commercial preparation is more sensitive to salt effects[5] than malt amylase is, and since, unlike salivary amylase, a stock preparation was available for use throughout the entire investigation. It was used as commercial pancreatin, a yellow, slightly hygroscopic powder obtained from Parke, Davis & Co. in 1922, and kept in a dark glass container in an ice box in this laboratory. In preparing the pancreatin for the experiments described in this paper 35 mg. were weighed out rapidly and transferred to a beaker. The powder was then rubbed to a paste with a few drops of a solution which had previously been prepared by mixing 5 cc. of N NaCl and 0.8 cc. of N 0.01 NaOH, diluting to 100 cc. with triply distilled water and chilling in the ice box. The remainder of the aqueous solution was then added; the dispersion was thoroughly mixed and filtered rapidly into a flask which was surrounded by ice.

Soluble starch prepared from potato starch by the Lintner method of treatment with dilute hydrochloric acid was used. The starch as purchased was further purified by washing repeatedly with distilled water and finally three times with triply distilled water. The moisture of the air-dried starch was determined, and enough of the starch was used in each starch dispersion to make 2 gm. of anhydrous starch in every 100 cc. portion used in the actual hydrolysis. In making the dispersion the required amount of starch was weighed into a tared

beaker, rubbed to a smooth, thin paste with triply distilled water, and poured into a comparatively large volume of hot triply-distilled water in an Erlenmeyer flask, brought quickly to the boiling point and boiled for three minutes. After cooling to room temperature the dispersion was diluted to volume with the required amounts of solutions of sodium chloride, sodium hydroxide and one of the salts under investigation, and with an additional amount of triply-distilled water.

All the salts used in this investigation were recrystallized twice from distilled water and once from triply-distilled water. They were air-dried with careful protection from dust and fumes. Their water-content was determined either in a one hundred degree oven or in a vacuum oven at lower temperature according to the stability of the salt. Solutions were prepared with amounts calculated as of anhydrous salt. Sodium chloride, sodium acetate and tertiary sodium citrate were used as such. Sodium borate was obtained in the final starch dispersion by adding recrystallized boric acid and bringing to the required pH with M 0.01 sodium hydroxide solution. Sodium phosphate was used in the early part of the investigation as the alkaline salt. When it was found advisable to increase the concentration of available phosphate from M 0.0005 to M 0.004, a mixture of alkaline and acid phosphates was prepared in order to avoid the use of large amounts of hydrochloric acid in securing the required pH. 500 cc. of the mixture contained 229.2 cc. M 0.4 Na_2HPO_4 and 270.8 cc. M 0.4 NaH_2PO_4. 2% starch dispersions containing M 0.05 Na Cl and enough of the "phosphate" mixture to give a potential phosphate-ion concentration of M 0.004 had pH values of approximately 6.8.

The water used in salt solutions and in starch or enzyme dispersions was triply distilled, that is, the ordinary distilled water of the laboratory was distilled a second time from alkaline permanganate solution; the distillate was transferred to a flask containing a small amount of phosphoric acid to hold ammonia and distilled a third time through a block tin condenser into a special bottle of resistance glass with avoidance of contact with the air. The third distillate was prepared as needed.

Method. The starch hydrolyses of this investigation were carried out in series of six to twelve members. In each series the 2% starch dispersions varied either in salt content or in hydrogen-ion concentration. In series in which the hydrogen-ion concentration was the variable, different amounts of N 0.01 NaOH (or HCl) were measured from a calibrated burette into graduated 100 cc. cylinders which had been calibrated for 62.5 cc. and 100 cc. Triply-distilled water was added to make a volume of 62.5 cc. and the solution was poured into a 200 cc. Erlenmeyer flask. A 62.5 cc. portion of 4% starch dispersion prepared as described above and containing the required amounts of sodium chloride and of one of the alkaline salts was measured in the same cylinder and transferred to the Erlenmeyer flask. The two portions were thoroughly mixed by pouring back and forth from flask to cylinder. Finally 100 cc. were kept in the cylinder for hydrolysis and 25 cc. were reserved in the flask at ice box temperature for determination of hydrogen-ion concentration. In series in which the hydrogen-ion concentration was fixed and the alkaline salt varied, a similar method of preparing the 2% starch dispersions for hydrolysis was used, but the alkaline salt in a solution of convenient molarity and the sodium hydroxide solution required to bring the final volume of 125 cc. to the required pH were measured into the cylinder, diluted to 62.5 cc. and mixed with 62.5 cc. of a 4% starch dispersion containing sodium chloride.

Cylinders containing the starch for hydrolysis were placed in a rack in a constant temperature bath regulated at 40° C. $\pm$ 0.05°. At least half an hour was allowed for the starch to reach the required temperature.

Meanwhile 1 cc. portions of the enzyme solution were measured with a calibrated 1 cc. pipette graduated to hundredths of a cubic centimeter into 200 cc. Erlenmeyer flasks which were kept cold by standing in ice. These flasks were then placed in a brass rack. The starch dispersions of the series were poured upon their corresponding enzyme portions at 15 second intervals. The rack was suspended in the bath and the hydrolysis was allowed to proceed for 30 minutes. The hydrolysis was stopped by adding 50 cc. portions of mixed

Fehling solution to the flasks of the series in the order of the starch additions and at 15 second intervals, so that each hydrolysis lasted exactly 30 minutes. The flasks in their rack were then transferred to a boiling water bath which was kept boiling vigorously for fifteen minutes.

The cuprous oxide precipitated by the reducing sugars formed in the hydrolysis of the starch was collected on the asbestos pads of Gooch crucibles, and washed with hot water, alcohol and ether. The crucibles were dried in an electric oven at 100° C. and weighed. The weight of cuprous oxide was obtained by difference. Crucibles were cleaned with 1 : 1 nitric acid and reweighed between each using.

Precautions. The greatest care was taken throughout the experiments to prevent contamination of the enzyme by any foreign material. All glassware was washed with hot soap suds and rinsed with hot tap water. It was rinsed with 1 : 1 nitric acid to remove possible traces of copper which inhibits the activity of the amylase markedly[33]. It was rinsed again with tap water, several times with distilled water and finally with triply-distilled water and then dried by inverting on glass plates. When not in use glassware was kept filled with water following the procedure of this laboratory which is based on the finding of Ford[34] that weathering of glass on exposure to air causes danger of introduction of alkali into the enzyme solutions. This method increased the ease with which glassware that had held starch preparations could be cleaned. Dishes were washed just long enough before using to allow for drying. The room was kept free from fumes and as free as possible from dust.

Since solutions of pancreatic amylase deteriorate much more rapidly at high temperatures than at low[35], water used in making the solution was always brought to ice-box temperature and all flasks receiving portions of the solution were chilled by surrounding with crushed ice.

The deteriorating effect of light was diminished by working in a north room. Window shades were drawn and no artificial light was used during the preparation of the enzyme

solution and during the hydrolysis. The regulating lamps in the bath were screened.

Hydrogen-ion determinations. All starch preparations used in hydrolyses were tested electrometrically for hydrogen-ion concentration. Calomel cells and hydrogen electrode holders were of the type described by Wilson and Kern[36]. A Wolff potentiometer was used with a standardized Weston cell as the standard of potential. A Leeds and Northrop recorder indicated when the system under investigation had reached equilibrium. A saturated calomel half-cell was used. The hydrogen electrodes were of platinum coated with platinum black. An electrode might be used for several readings on the same day but was never used on a second day. Electrodes and calomel cell were checked against standardized tenth-normal hydrochloric acid before each determination. Solutions to be tested were used in approximately 5 cc. portions in vessels of small diameter. Determinations were made at room temperature. Hydrogen-ion concentration was calculated as pH by means of the equation:

$$pH = \log \frac{1}{[H^+]} = \frac{\text{E.M.F. (observed)} - \text{E (calomel electrode)}}{0.0001983\ T}$$

RESULTS

Many preliminary tests were made with starch dispersions containing M 0.0005 H_3BO_3. It was found that at this molarity hydrogen-ion concentrations could not be duplicated from day to day and that in making electrometric determinations it was difficult to secure equilibrium in the solution. Increase of molar concentration to M 0.002 gave more consistent results with the borate preparations but did not eliminate difficulty with starch dispersions containing sodium acetate. It was finally decided to use the alkaline salts at a concentration of M 0.004. Duplication of hydrogen-ion concentration was then quite certain with all the salts except sodium acetate.

The latter salt never gave satisfaction from the practical view-point of duplication of results. Four series of hydrolyses

in which the pH of starch-acetate preparations varied failed to show a definite optimum pH. Solutions made up on different days with equivalent amounts of sodium hydroxide showed pH values one to four tenths lower at one time than at another. This made it practically impossible to compare the hydrolysis of starch in presence of acetate with that in presence of other alkaline salts at the same pH. In six comparisons under the same conditions and at pH values which did not differ by more than 0.2 (in the neighborhood of pH 7.0) acetate-starch yielded 4, 9, 11, 18, 31, and 45 mg. less cuprous oxide than the standard phosphate-starch. Work with sodium acetate was discontinued at this point.

Optimum pH for starch hydrolysis in the presence of each salt. A preliminary study of the optima in pH for starch preparations containing the different alkaline salts at M 0.004 showed an optimum for each salt in the neighborhood of pH 7.1. The optima were not sharp however. Usually the yields of cuprous oxide at 7.0 or 7.2 were slightly lower than at pH 7.1 ; occasionally they were equal or even slightly higher. Experimental variations are unavoidable in working with a substance as sensitive to changes in light and temperature as pancreatic amylase. To eliminate the effect of experimental errors each salt was used in an extended series of hydrolyses in which the effects of the three pH values were compared. Results are given in Table I. In each series a weight of cuprous oxide obtained at one pH is paired with one obtained at another pH under the same experimental conditions. pH 7.1 is compared with both 7.0 and 7.2 and the latter two are compared for a check upon the differences. It will be noted that with each salt the highest yield is obtained at pH 7.1 but that the differences between 7.1 and 7.0 or 7.2 are so small and carry such large probable errors that they are insignificant for practical laboratory work.

TABLE I.

The Optimum pH for the Activity of Pancreatic Amylase in the Hydrolysis of Starch Dispersions containing Different Anions. (Mean Weight of Cuprous Oxide in Milligrams.)

Anion	No. of observations	pH 7.0	pH 7.1	pH 7.2
Phosphate	8	329.9±3.2	332.3±2.5 excess 2.4±4.0	
	10		332.8±2.0 excess 0.6±3.3	332.2±2.6
	12	327.0±2.6		329.6±2.5 excess 2.6±3.6
Citrate	9	345.2±2.9	346.6±3.0 excess 1.4±4.2	
	12		342.0±2.1 excess 4.0±3.1	338.0±2.3
	10	341.5±2.5 excess 0.2±3.2		341.3±2.0
Borate	11	325.4±1.3	327.7±1.3 excess 2.3±1.9	
	9		327.9±1.9 excess 2.3±2.1	325.6±0.8
	7	322.0±1.7		325.4±1.0 excess 3.4±2.0

Effect of changing the concentration of the alkaline salt. Boric acid was varied in molar concentration in starch dispersions through the range 0.0005, 0.001, 0.002, 0.004, 0.007 and 0.01. The dispersions were brought to practically identical pH values, 6.9-7.0, with sodium hydroxide and hydrolyzed under the same experimental conditions. Excluding the lowest member of the series, it was found that the maximum difference in the weight of cuprous oxide obtained was five milligrams. At the lowest concentration of borate, M 0.0005, 9-14 mg. less cuprous oxide were obtained. Since attainment of equilibrium during electrometric determination of the hydrogen-ion con-

centration of other solutions containing this low amount of borate had been difficult, and since this solution contained the same amount of sodium hydroxide as the next higher member of the series, it seems probable that the pH of the solution was actually higher than 7.0. Deviation from optimum pH would account for the lower yield of cuprous oxide. The other members of the series indicate clearly that a tenfold increase in borate concentration has no effect upon the extent of hydrolysis of the starch.

A similar series of experiments with a wider range of concentration was made with the phosphate mixture described above. Preliminary tests with the highest concentration, M 0.05, in four series, in which the pH was the variable, indicated that higher yields of cuprous oxide would be obtained at 7.0 than at 7.2. Both of these points were included therefore in the comparison of hydrolyses in the presence of different amounts of phosphate. Three comparisons confirmed the opinion that hydrolyses in the presence of M 0.05 "phosphate," unlike those with M 0.004 "phosphate," are appreciably affected by a change in pH from 7.0 to 7.2. The mean yields of seven hydrolyses at each pH were 340.7 ± 3.1 mg. Cu_2O at 7.0 and 332.7 ± 3.0 mg. at 7.2 with a difference in favor of pH 7.0 of 8.0 ± 4.3 mg.

The effect of varying the "phosphate" concentration is shown in Table II. At pH 7.0 the yields of cuprous oxide are practically identical in the presence of M 0.004 "phosphate" and M 0.01 "phosphate"; at pH 7.2 also yields are nearly the same at the two concentrations. The latter pH is slightly more favorable to the hydrolysis than pH 7.0. With M 0.05 "phosphate" however 7.0 is the more favorable pH. At this value the yield of cuprous oxide is practically identical with that obtained at pH 7.2 with the lower concentrations. Apparently therefore a twelve-fold change in concentration of the alkaline salt makes no difference in the activity of pancreatic amylase if the hydrogen-ion concentrations of the starch dispersions as determined at room temperature are adjusted to the optimum for each salt concentration.

At the lowest concentration of phosphate used in these comparisons, M 0.0005, yields of cuprous oxide obtained in individual experiments may be as high as those obtained at higher concentrations. As a rule, however, yields are lower. In this connection it must be noted that solutions containing M 0.0005 phosphate are strongly affected by slight changes in the amount of sodium hydroxide introduced in attempting to bring them to the desired pH value. In these experiments 4.6 cc. N/100 NaOH in 125 cc. of starch dispersion have given a pH value of 7.2 whereas 4.7 cc. have given pH 7.3. In contrast, at the concentration at which most of the tests of this inves-

TABLE II.

Cuprous Oxide Yields with Varying Phosphate Concentration. (pH 7.0 and 7.2)*

	M 0.0005		M 0.004		M 0.01		M 0.05	
Expt.	pH 7.0	7.2	7.0	7.2	7.0	7.2	7.0	7.2
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	(295)[1]	313	315	320	320	318	321	315
2	343	(341)[2]	350	(351)[3]	343	351	353	345
3	307	325	(326)[4]	333	328	336	331	326
Av.	315.0	326.3	330.3	334.7	329.7	335.7	335.0	328.7

* Figures in parentheses indicate yields obtained at pH values which differed slightly from those at head of column.
(1) pH 6.8; (2) pH 7.3; (3) pH 7.3; (4) pH 6.9.

tigation were made, M 0.004, 3 to 4 cc. of N/100 NaOH have been needed to bring about a change of one-tenth in pH, and ten times more has been needed for the same pH change in the presence of M 0.05 phosphate mixture.

In the ordinary work of the laboratory the preparation of starch dispersions may involve slight volume errors and will necessitate exposure to the air and the consequent possibility of solution of carbon dioxide during mixing and hydrolyzing. These factors would change the hydrogen-ion concentration of dispersions if not adequately buffered. The figures given above are typical of the experience of this investigation and show that disodium phosphate at a concentration of M 0.0005

is not a satisfactory buffer, a fact which accounts for the inconsistent results obtained with it.

The value of the alkaline salts in starch hydrolysis. The comparison of the effects of the three alkaline salts was planned to show whether or not any one of the three characteristic anions had a specific activating (or inhibiting) effect upon the hydrolysis of starch. Table III shows the yields of cuprous oxide in a series of experiments in which each hydrolysis in the presence of one salt was paralleled by an hydrolysis in the presence of each of the other salts. For each group the enzyme solution and the stock starch dispersion were identical and experimental conditions were as nearly alike as possible. Within the groups the pH did not vary by more than one-tenth in the neighborhood of 7.1, a variation which has been shown to be without significant effect upon the extent of the hydrolysis.

The mean yields of cuprous oxide for each group of twenty-three hydrolyses were 335.3 ± 1.1 mg. for phosphate-starch, 332.8 ± 1.3 mg. for citrate-starch and 322.3 ± 1.4 mg. for borate-starch. The mean yield in the presence of phosphate was 2.5 ± 1.7 mg. higher than in the presence of citrate. This difference is so small and carries such a large probable error that it can not be attributed definitely to a specific activating effect of the phosphate anion upon enzyme, or starch-enzyme complex.

The yield with borate is markedly lower than with either citrate or phosphate. This fact can not be attributed to an inhibiting effect of the anion since the results given above have shown that a ten-fold increase in borate concentration had no effect upon the extent of hydrolysis of starch by pancreatic amylase.

TABLE III.

Comparison of Yields of Cuprous Oxide when Pancreatic Amylase Acts in Presence of Three Alkaline Salts at pH of approximately 7.1.

Phosphate	Citrate	Borate
340 mg.	331 mg.	324 mg.
340	329	323
345	341	328
342	343	332
342	328	326
339	346	338
335	332	323
331	331	323
342	339	329
339	335	327
326	330	320
331	334	319
330	333	321
335	331	317
320	327	308
319	321	301
323	306	315
326	318	297
339	339	330
342	343	330
341	335	330
341	342	324
—	—	—
335.3 ± 1.1	332.8 ± 1.3	322.3 ± 1.4
standard deviation 8.0	standard deviation 9.1	standard deviation 9.7

excess

phosphate over citrate	2.5 ± 1.7
citrate over borate	10.5 ± 1.9
phosphate over borate	13.0 ± 1.8

Table III also shows the standard deviation from the mean yield of cuprous oxide with each salt. This is the measure of the variability of the yield. The standard deviation is large in each case and increases in the order phosphate, citrate and borate. It reflects not only the difficulties involved in weighing out the slightly hygroscopic pancreatin and in making up

and filtering the solution, but also the sensitiveness of the enzyme in solution to changes in light and temperature and acidity. Starch dispersions containing each of the anions were subjected to the same variations in light and temperature. They were not however subjected to the same changes in hydrogen-ion concentration. Borate-starch was less fully protected against change than either citrate or phosphate preparations. In experiments in the course of this investigation a change of one-tenth in pH value was produced in starch preparations containing M 0.004 H_3BO_3 upon increasing the amount of added sodium hydroxide (N/100) by approximately 0.4 cc. The same change in starch dispersions with M 0.004 $Na_3C_6H_5O_7$ required twice as much sodium hydroxide, and with M 0.004 phosphate mixture eight times as much.

Such results would be expected in the light of the work of Sörensen[30] and of Clark[37]. Their investigations have shown very definitely that a solution containing sodium borate and boric acid is not capable of maintaining a definite pH value in the neighborhood of 7.0 upon addition of small amounts of sodium hydroxide. At this pH they found that sodium citrate solutions were able to maintain pH values to a slight extent and that sodium phosphate solutions were excellent "buffers."

Effect of omission of alkaline salt from starch dispersions. To obtain further information about the function of the alkaline salts, hydrolyses were carried out in the presence of sodium chloride either with or without sodium phosphate mixture. In all cases the salts mentioned were the only electrolytes added except the sodium hydroxide used in small amount to secure the required pH. With sodium chloride alone there was no assurance that pH 7.1 is the optimum for the hydrolysis. Therefore the amounts of added sodium hydroxide were varied to give a range of pH values. As Clark[38] has pointed out, unless a solution is buffered to some extent in some way, it is almost impossible to make an accurate electrometric determination of the pH. Beans and Hammett[39] have also found that the measurement of hydrogen-electrode potentials in unbuffered salt solutions using the ordinary technique of the bubbling electrode is absolutely uncertain unless special pre-

cautions are taken in the preparation and handling of platinized electrodes. Inconsistent values, varying from pH 6.4 to pH 7.6, were obtained for the series of solutions (Experiment I, Table IV) in which the starch itself was the only buffer. The two solutions to which the smallest and the largest amounts of sodium hydroxide had been added gave low yields of cuprous oxide; intermediate solutions gave 2-6 mg. less than a standard starch dispersion which contained phosphate anion and which was hydrolyzed by means of a portion of the same enzyme solution under the same experimental conditions.

The amount of sodium hydroxide present in that starch dispersion of the above series which yielded the most cuprous oxide, and thus showed greatest saccharogenic activity of the enzyme, was used in two experiments in which starch prepared with sodium chloride was compared with that containing phosphate as well. Results are shown in Table IV. The enzyme solution in Experiment 3 contained less pancreatin than the other enzyme solutions used throughout the investigation.

TABLE IV.

Comparison of Hydrolyses with and without Phosphate.

Experiment	NaCl	Phosphate	cc. NaOH	pH	mg. Cu_2O
1	M 0.05	——	5.0 N/100	6.4*	317
	"	——	5.8 "	6.8*	331
	"	——	6.1 "	7.1*	333
	"	——	6.4 "	7.0*	329
	"	——	6.7 "	7.6*	332
	"	——	7.3 "	7.5*	313
	"	M 0.004	15.0 "	7.2	335
2	"	——	6.1 "	6.9*	333
	"	——	6.1 "	6.9*	330
	"	M 0.05	7.5 N/10	7.0	342
3	"	——	6.1 N/100	6.8*	208
	"	——	6.1 "	7.0*	207
	"	M 0.05	7.5 N/10	7.0	204

* unreliable, since solution was buffered by starch only.

The influence of sodium chloride upon the hydrolysis. After consideration of the work of Brönsted upon the abnormalities in activity coefficients of ions due to the presence of relatively large amounts of neutral salts, it was decided to attempt a comparison of the activating effects of borate, citrate and phosphate anions upon the hydrolysis of starch in the absence of sodium chloride.

Table V shows the results of a preliminary study of the effect of omitting sodium chloride from starch dispersions. In Experiment 4 direct comparison was made of the extent of hydrolysis in a starch dispersion containing M 0.05 phosphate and no sodium chloride with that in one containing phosphate and chloride both at M 0.05. Addition of sodium chloride caused an eight-fold increase in the amount of cuprous oxide precipitated. In Experiment 3 the phosphate concentration was M 0.004. The only sodium chloride present was the trace added as impurity in the commercial pancreatin. The yield of cuprous oxide, 40 to 45 mg., was little higher than the 36 mg. "blank" obtained when 100 cc. of a 2% starch paste containing salts but no enzyme acted upon Fehling solution.

These results showed that a comparison of the effects of the salts under investigation would be impossible in the absence of sodium chloride. They led however to a further study of the influence of sodium chloride upon pancreatic activity.

A comparison of the low result of Experiment 4 in which phosphate was the electrolyte added to both enzyme and starch preparations with the first two hydrolyses in Experiment 5 in which sodium chloride was the electrolyte shows that phosphate and chloride are not interchangeable in the hydrolysis as a whole. Therefore both salts are not acting merely as electrolytes.

Experiments 1 and 2 show that phosphate may be substituted for chloride in the enzyme solutions. This means simply that in the time between the dissolving of the pancreatin in ice-cold water and the addition of starch to 1 cc. portions of the cold enzyme solution, approximately three quarters of an hour, deterioration of the amylase is not appreciably faster in the presence of one salt than in the presence of the other.

TABLE V.

Influence of Sodium Chloride and Sodium Phosphate.

Experiment No.	Enzyme Solution NaCl	Enzyme Solution Phosphate	Enzyme Solution pH	Starch Dispersion NaCl	Starch Dispersion Phosphate	Starch Dispersion pH	Cu_2O mg.
1	—	0.05	6.9	0.05	0.05	7.0	360
	0.05	—	6.4a	0.05	0.05	7.0	342
2	—	0.05	6.9	0.05	0.05	7.0	249
	0.05	—	7.0	0.05	0.05	7.0	204
3	—	0.0005	7.3	—	0.004	7.0-7.5	40-43b
4	—	0.05	6.9	—	0.05	6.9	45
				0.05	0.05	7.0	360
5	0.05	—	6.4	0.05	—	6.9	333
				0.05	—	6.9	330
				—	0.05	6.8	175
				0.05	0.05	7.0	342
6	0.05	—	7.0	—	0.05	6.9	119
				0.05	0.05	7.0	204
7	0.05	—	6.6	—	0.004	7.2	146
				—	0.004	7.4	128
				0.05	0.004	7.2	342
				0.05	0.004	7.2	339

a. Enzyme solutions buffered by the pancreatin material itself give steady readings on the potentiometer. They are not sufficiently buffered to allow duplication of pH values from day to day. Twenty-four different determinations show a mean pH value of 6.6 $\pm$ 0.03 with a maximum range of 6.3 to 7.1. If the pH of the cold enzyme solution during the time required for filtering and pipetting affects the activity of the enzyme, change is so small as to be masked by variations in pancreatin content.

b. Seven hydrolyses.

In Experiments 5 and 7 cuprous oxide precipitates weighing 175, 146 and 128 mg. were obtained from hydrolytic products of starch dispersions in which the only sodium chloride present was that introduced with the enzyme solution. 1 cc. of the latter containing M 0.05 NaCl was added to 100 cc. of

starch dispersion, thus giving a final concentration of sodium chloride of approximately M 0.0005. In contrast only 41 mg. of cuprous oxide were obtained from an hydrolysis in which the starch dispersion contained M 0.0005 NaCl. It is interesting to note the greater "activating" power of the sodium chloride in the former case in which higher concentration of the amylase and lower viscosity of the medium allow greater opportunity for contact of enzyme and salt.

CONCLUSIONS

Pancreatic amylase acting for 30 minutes at 40° C. upon 2% starch dispersions containing M 0.05 NaCl and the sodium salts of either citric, phosphoric or boric acids at M 0.004 is most active when the pH value of the starch dispersions, as determined electrometrically at room temperature, lies in the range pH 7.0-7.2. A value of 7.1 may possibly be more favorable than either 7.0 or 7.2.

A change from M 0.004 to M 0.05 in the concentration of phosphate added to the starch as a mixture of disodium and monosodium phosphates lowers the optimum pH value slightly and shortens the optimum range. At the higher concentration of phosphate pH 7.0 is more favorable to the enzyme action than pH 7.2.

Change in concentration of "phosphate" from M 0.004 to M 0.05 or of borate from M 0.001 to M 0.01 does not change the activity of the enzyme if the starch dispersions are brought to that pH value which is optimum for each concentration.

Slightly lower yields of the reducing sugars at the lowest concentration of the alkaline salts which has been studied (M, 0.0005) may be attributed to inability of the salts at this concentration to maintain a definite pH throughout the hydrolysis.

The extent of hydrolysis of starch dispersions prepared with phosphate is slightly greater than that of starch dispersions prepared with citrate and markedly greater than that with borate. The latter finding is probably due to the recognized inability of sodium borate-boric acid solutions to act effectively as buffers at pH 7.

Activity of the enzyme is practically the same in starch dispersions in which sodium chloride and sodium hydroxide are the only added electrolytes as it is in starch dispersions containing sodium phosphate as well.

A mixture of alkaline and acid phosphates having an equivalent molar concentration of electrolyte can not be substituted for sodium chloride in the hydrolysis of starch by pancreatic amylase.

In view of the fact that hydrolyses in the absence of sodium phosphate may proceed as far as those in its presence and that results are nearly the same with phosphate and citrate, it is improbable that the phosphate or citrate salts exert any specific effect upon the hydrolysis. Their influence is apparently that of buffers protecting the starch dispersions from slight changes in hydrogen-ion concentration tending to occur in the course of hydrolysis.

The greater effectiveness of phosphate mixtures as buffers makes it desirable for consistent results in the laboratory that a phosphate buffer mixture at a concentration of at least M 0.004 be introduced into the starch dispersions.

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